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13. ABSTRACT *(Maximum 200 Words)*

The aetiology of Gulf-War (GW) related illnesses remains unclear. The proposal has been made that multiple vaccines, given under the stress of deployment with additional effects of the T helper 2 (Th2) adjuvant pertussis could skew the immune response towards a Th2 profile. The Th2 profile, characterised by the cytokines interleukin-4 (IL-4) and IL-10, has been associated with syndromes such as allergic disease, hypersensitivity and depression, symptoms characteristic of which are often reported by sick GW veterans (GWVs). We studied whether self-reported illness in GWVs is associated with a Th2 shift by examining Th2 (IL-4, IL-10) and Th1 (IL-2, interferon-γ) intracellular cytokine staining in helper T lymphocytes by flow cytometry after mitogenic stimulation. We studied GWVs with and without illness, as well as a group of control servicemen with similar symptoms who were either not deployed (era controls) or deployed to the Bosnia conflict. We find changes in cytokine profiles in servicemen reporting multi-symptom illness. Our data suggest that deployment to the Gulf and multi-symptom illness are associated with immune activation.

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Table of Contents

Introduction

In this study, we set out to examine the relationship between immunological markers of Th1/Th2 cytokine balance, biological warfare vaccines, multiple vaccination and Gulf War related illness.

Soon after their return from deployment, it became apparent that members of the armed forces who served in the Persian Gulf War in 1990 were reporting a number of unexplained illnesses, including symptoms typical of the chronic fatigue syndrome (CFS). In order to clarify whether such an increase in morbidity was real, and the precise nature of the symptoms involved, we undertook an epidemiological study of the prevalence of unexplained illness in the population at risk using a two stage design. This work, which was also US AMRMC funded, reported its first stage findings in 1999. These findings were the result of a questionnaire based study in which we compared 2961 United Kingdom service personnel who served in the Gulf conflict with 2620 who served in Bosnia, and 2614 who were in the military during the time of the Gulf War but served in neither theatre ("Era"). All were chosen randomly. The results (Unwin et al, 1999) showed that service in the Gulf was associated with a range of adverse, self reported outcomes. Symptoms such as fatigue, irritability, headache and difficulties with sleep were about three times commoner in the Gulf group. Although we were unable to confirm that these results indicated a unique "Gulf War syndrome" (Ismail et al, 1999), we concluded that this was unequivocal evidence that Gulf service had led to a subjective decline in well being and symptomatic ill health amongst those who served in that theatre.

The next question we addressed was possible aetiology. This was examined using the same cohorts, and accessing their self-reported exposure to potential aetiological agents, but especially vaccines. We found (Hotopf et al, 2000) that receipt of multiple vaccines during deployment was associated with 5 out of 6 disease outcomes (multi-symptom illness (CDC defined) fatigue, psychological distress, health perception and physical functioning but not post-traumatic stress reaction), with the strongest association for multi-symptom illness (odds ratio 5.0). Since administration of multiple vaccines *before* deployment was only associated with one outcome (post-traumatic stress reaction), our results supported the concept that multiple vaccinations combined with the stress of deployment may be associated with adverse health outcomes.

These findings are consistent with the so-called "Rook and Zumla hypothesis", namely that multi-symptom illness in war veterans is the result of a shift in cytokine balance from T helper 1 (Th1) to Th2 (Rook & Zumla, 1997). The hypothesis is based on a number of observations. First, GWVs received multiple vaccinations administered within a short space of time. It is well documented that antigen load can deviate the immune response towards Th2 (Bretscher et al, 1992; Hernandez-Pando et al, 1994). Second, vaccines were administered in preparation for war, and included those against biological warfare agents such as *Bacillus anthracis* (anthrax) and *Yersinia pestis* (plague), which were very real threats during this conflict. It is propososed that the resultant high levels of stress in vaccine recipients leads to elevated levels of circulating levels of the

hormone cortisol, which has been shown under many experimental conditions to deviate the immune response towards Th2 (Wu et al, 1991; Brinkman et al, 1995; Padgett et al, 1995; Ramirez et al, 1996). Finally, although natural infection with *Bordetella pertussis* and its whole cell-derived vaccine promote a strong Th1 response, paradoxically the acellular vaccine component pertussis toxin used as adjuvant in GW vaccinations causes Th2 deviation (Munoz et al, 1990; Mu et al, 1993). These proposals take on a greater significance when considering the symptoms and immunopathological findings in chronic fatigue syndrome, which bear many striking similarities with Gulf War associated illness and could be interpreted as representing a switch to a Th2 cytokine profile as has been suggested (Rook & Zumla, 1997).

The Th1/Th2 paradigm of immune regulation, and its relationship to immune-mediated human diseases has become increasingly accepted in recent years. Stated briefly, polarised Th1 cells secrete immune activating cytokines such as interferon-γ, (IFN-γ) and promote cell-mediated immune responses (eg killing of intracellular parasites). Polarised Th2 cells secrete cytokines such as interleukin-4 (IL-4), IL-5 and IL-10. In recent years, and since the original design of our study, it has also become clear that IL-10, a cytokine with a predominantly down-regulatory effect on immune responses, may not only be secreted by Th2 cells, but by T cells termed regulatory, or Treg. Th2 cytokines have a tendency for mutual inhibition, potentially creating conditions under which subtle disturbances can lead to pathological imbalance over a period of time. An imbalance of Th2 over Th1 immunity is associated with allergic disease and hypersensitivity (reviewed in Abbas et al, 1996) and mood changes including depression (reviewed in Rook & Zumla, 1997). These observations form the basis of the proposal by Rook and Zumla that such an imbalance could be associated with Gulf War-related illness. In recent years it has become possible to measure the products (cytokines) of Th1 and Th2 lymphocytes in peripheral blood with great sensitivity and accuracy. In some disorders, such measurements correlate very closely with the proposed immune pathogenesis of the disease. In allergy, for example, a preponderance of Th2 activity is observed in peripheral blood (Romagnani, 1994), consistent with the fact that Th2 cytokines promote the production of allergen-specific IgE and eosinophil recruitment, the two main immunopathological hallmarks of clinical allergy.

There is no single bio-assay to establish the Th1/Th2 balance in an individual. The current "state of the art" suggests that a multi-layered approach should be employed initially, to identify a set or sets of examinations which provide meaningful data. In our Statement of Work we proposed to begin with an examination of the intracellular production of cytokines within mitogenically stimulated CD4 and CD8 T cells. The establishment and validation of this technique, and the results from this study form the major part of this report.

Body

Patients

The blood samples in this study were drawn from a subset of veterans in the previously reported large epidemiological study (Unwin et al, 1999). These stage 2 samples were randomly selected from veterans who participated in the stage 1 study. We have selected at random a sample of sick Gulf veterans, defined as all those veterans who scored 72.2 or less on the SF-36 physical functioning subscale. This was the cut-off value for the lowest 10th centile of the distribution of the SF 36 PF in the Era (baseline group). There are two control groups. Similar numbers of sick veterans of the Bosnia conflict or sick service personnel who were not deployed in either conflict (era controls) and fulfill the same criteria for illness have been recruited, as have similar numbers of well Gulf veterans. Tests carried out in conjunction with sampling, and which form the basis of the stage 2 study, include clinical assessments, psychiatric and neuropsychiatric screening, respiratory function, and also in a sub group intensive neurophysiological and neuromuscular investigations. All subjects are also asked to bring their vaccination records with them where available, and to consent to medical record review. In addition, with consent, we have acquired medical records in order to obtain definitive information regarding vaccine status for each participant.

Blood samples, drawn from epidemiologically defined cohorts, were processed and analysed by an individual lab worker blinded to the source of the sample. The Gulf veterans in the study had seen service in the UK military in the Gulf region between September 1, 1990 and June 30, 1991. We studied 57 patients who are without any symptoms of the "Gulf War related illness" (Gulf well veterans - GWV) and 63 veterans who are ill. As a first matching control group were 20 randomly chosen veterans who had served in Bosnia between April 1, 1992, and February 6, 1997 and 38 personnel serving in the armed forces on January 1, 1991, who were not deployed to the Gulf War (Era cohort). The Era cohort control group was matched for sex, age, rank, and fitness service status, while the Bosnia control group was matched only for sex, age, rank because the service status and fitness data were not available.

Methods adopted for determination of intracellular cytokine secretion

Heparinised whole venous blood was used for the in vitro cell stimulation, in order to observe the whole potential of cytokine production by lymphocytes. This method avoids partial activation of lymphocytes arising from the Ficoll-Hypaque gradient separation procedure. The whole blood culture prevents the unintentional separation of neighboring cells and serum components so that they retain their in vivo characteristics. Cytokine production in whole blood systems has also been reported to be greater than in purified PBMC preparations (De Groote et al, 1992).

We evaluated in an initial pilot study several protocols for the measurement of intracellular cytokine staining based on published papers. These included the assessment of various combinations of: mitogens (phorbol myristate acetate (PMA), phytohaemaglutinin (PHA), ionomycin, anti-CD3, and anti-CD28). We also examined which concentration of the various mitogens offered the greatest sensitivity over background (signal/noise ratio). As a result we chose to use in the main study the

polyclonal stimulants PMA and ionomycin as mitogens. These gave the best yield of cytokine production by lymphocytes compared to the other stimulants investigated. In previous studies PMA and ionomycin were also shown to be superior (Saner et al, 1996; North et al, 1996). In the study of intracellular cytokine expression, blockers of protein secretion are added in order to retain cytokines within the cell. We investigated the protein secretion inhibitors monensin and Brefeldin A added either immediately or 1-2 hours after stimulation. Monensin is an inhibitor of trans-Golgi function, while Brefeldin A inhibits protein transport between the endoplasmic reticulum and the Golgi apparatus. In the literature these two inhibitors used alone have produced inconsistent results (Jung et al, 1993; Prussin & Metcalfe, 1995; Nylander & Kalies, 1999). We adopted their use in combination, added immediately for the main study as this proved to provide the most effective yield of cytokines. Finally, we investigated in pilot trials the length of culture (6, 16, 24 hours). It is well known that each cytokine has different kinetics of intracellular expression so that they reach their respective maxima at different time points. The literature is inconclusive, with optimal values varying from 4 hours for IL-4 up to 64 hours for IFN-γ. In our own pilot evaluation we found that a 16-hour incubation period gave effective results for all of the 4 cytokines we were investigating.
In the main study therefore we cultured whole blood for 16 hours in the presence of Brefeldin A, monensin and the polyclonal activators PMA and ionomycin, followed by staining of CD4+ and CD8+ cells for IL-4, IFN-γ, IL-2, IL-10.

Heparinised whole blood was mixed with culture medium (RPMI1640 + 10%FCS + 2mM L-glutamine + 100IU/ml penicillin + 100μg/ml streptomycin) in proportion: 1 part blood to 2.6 parts of culture medium. The mixture was placed in 1ml aliquots into separate wells on a 24-well TC plate. One set of the wells was then supplemented with 5ng/ml of PMA and 745ng/ml of ionomycin. Protein secretion inhibitors Brefeldin A (5μg/ml) and monensin (2.08mg/ml) were added and blood cultured at 37°C for 16 hours in an atmosphere of 95% air and 5% CO_2. As a negative control we also measured the intracellular cytokine production from a duplicate set of cultures in wells containing resting cells. These contained no polyclonal stimulators and were incubated with the protein blockers Brefeldin A and monensin only.
The cells were then harvested, washed and stained with PerCP conjugated anti-human CD4 monoclonal Antibodies (mAb) and FITC-conjugated anti-human CD8 (both from Becton Dickinson) in order to phenotype the cells of interest. Then the cells were washed 2 times with PBS containing 5% FCS. The intracellular cytokine staining protocol was followed as suggested by the manufacturer (Caltag Lab) of the Fix&Perm Permeablilization Kit used in our studies. In brief, cells were fixed with Reagent A (Fix&Perm Permeablilization Kit) and incubated for 15 minutes at room temperature in the dark. After washing 2 times the Reagent B was added (as a permeabilization solution) along with appropriate PE-conjugated anti-cytokine (IL-2, IFNγ, IL-4, IL-10) mAbs or fluorochrome and isotype-matched mAbs as controls (all from Pharmingen) and incubated at 4°C for 30 minutes. Finally, the stained cells were washed and suspended in 200μl of PBS for flow cytometry analysis.

Intracellular cytokine production by CD4 and CD8 cells was assessed. Ten thousand events of gated lymphocytes (on forward and side scatter) were acquired and analyzed using CELLQuest software (Becton Dickinson). Dot plot quadrant statistics were set on the basis of corresponding isotype-matched control mAb during data analysis such that the frequencies of CD4 or CD8 positive cell populations capable of IL-2, IFN-γ, IL-4, IL-10 production were determined.

Results

The results for this study have been written up and were first submitted for publication on February 12 2002:

T helper 2 type immune activation in Gulf War veterans with multi-symptom illness Anna Skowera, Matthew Hotopf, Elżbieta Sawicka, Ruben Varela-Calvino, Catherine Unwin, Vasilis Nikolaou, Lisa Hull, Khalida Ismail, Anthony S David, Simon C Wessely, Mark Peakman. Submitted.

A decision is awaited. The manuscript submitted is appended (Appendix VIII), and will be referred to in the results section below.

The results not included in the manuscript are shown in tables1-4 and figures 1 and 2 in the appendices, pages 15-20. In interpreting the results, we take the view that cells which demonstrate the presence of intracellular cytokines without stimulation represent a *recently activated* population. Cells which require mitogenic stimulation to reveal cytokine secretion represent *resting memory* cells. These two populations are considered separately.

1. Cytokine production by resting (recently activated) cells (appx I-VII)

CD4 T cell interleukin-4 secretion. Resting (unstimulated) IL-4 secretion is typically present at very low levels. Similar low levels were seen in the sick Bosnia/Era cohort and in the well GWVs. However, significantly elevated levels of resting IL-4 secreting CD4 T cells were seen in sick GWVs compared with the other service groups (p=0.0002 and 0.0007).

CD4 T cell interferon-γ secretion. Resting (unstimulated) IFN-γ secretion followed a similar pattern to IL-4, in that levels were only significantly raised in sick GWVs compared with sick Bosnia/Era cohort (p=0.0129) and well GWVs (p=0.0091).

CD4 T cell interleukin-2 secretion. Again, resting (unstimulated) IL-2 secretion followed a similar pattern to IL-4 and IFN-γ, in that levels were only elevated in sick GWVs compared with Bosnia/Era cohort (p=0.0017) and well GWVs (p=0.0054).

CD4 T cell interleukin-10 secretion. Resting IL-10 secretion was similar in all groups.

Changes in intracellular cytokine expression by CD8 cells were similar to those seen in CD4 cells, but had a less distinct pattern (figure 2).

We interpret these data as showing that, at the time of blood sampling, GWVs with multi-symptom illness show on-going immune activation, as evidenced by an elevation of cytokine+ T cell numbers. Both Th1 and Th2 cytokine+ cells were elevated, suggesting that the acute, on-going activation does not follow any specific pattern of Th1/Th2 skewing.

Analysis of clinical parameters and vaccine records show no significant relationship between these immune changes in resting cells and any clinical measure, symptom, single vaccine, or multiple vaccines.

2. Cytokine production by activated (memory) cells (appx VIII)

The results obtained for activated CD4+ T cell expression of cytokines are given iin Appendix VIII, as a copy of the manuscript recently submitted. The results section is reproduced verbatim below, and therefore figures and tables referred to may be found in appendix VIII.

2.1 Th1/Th2 balance in Gulf War veterans

We found evidence for a shift towards Th2 immunity in symptomatic GWVs. Mean levels of IL-4 producing Th2 cells were higher in symptomatic GWVs than in well GWVs ($p<0.05$) and symptomatic BEVs ($p<0.05$) (figure 1, table 1). Mean levels of IL-10 producing CD4 T cells were similar in symptomatic GWVs and sBEVs, but were higher in both than well GWVs ($p=0.001$ and $p<0.05$, respectively). In contrast, levels of IFN-γ and IL-2 producing Th1 cells were similar in all study groups.

To confirm that IL-4 producing CD4 T cells were indeed Th2 and not Th0 (Th0 cells secrete IL-4 and IFN-γ), a subset of cases ($n=40$) was also analysed by 4-colour flow cytometry for co-secretion of IL-4 and IFN-γ. In all cases, over 85% of IL-4+ CD4 T cells did not secrete IFN-γ, confirming the predominance of Th2 cells. There was no correlation between levels of IL-4 producing and IL-10 producing CD4 T cells in sGWVs, suggesting the existence of two distinct, expanded populations of effector memory cells.

2.2 Illness effects on Th1/Th2 balance in Gulf War veterans

Our data suggest that sickness amongst GWVs is associated with elevated levels of IL-4+ Th2 cells. To examine the effects of illness on Th1/Th2 balance, we compared results between symptomatic and well GWVs. No single symptom was significantly associated with Th2 skewing but when levels of IL-4+ and IL-10+ cells were controlled for mood (assessed using the BDI), significance levels were reduced, becoming non-significant in the case of IL-4 (Table 1). These results suggest that expansion of IL-4+ and IL-10+ Th2-type cells is associated with depression, but this is not a sufficient explanation of the observed findings.

During the period between the initial stage I questionnaire based study, and clinical assessment carried out for stage II, which included the blood sampling for the current

study, 25 (42%) sGWVs showed sufficient improvement in their SF36 physical functioning score that they no longer fulfilled our criteria to be considered "ill", and 3 (6%) of the wGWVs had experienced a deterioration in their SF36 physical functioning and were considered cases. When these cross-overs were taken into account, the association between illness and Th1/Th2 balance also changed. Although mean levels of IL-4+ cells remained higher in symptomatic GWVs, this difference was no longer significant (Table 2). However, mean levels of IL-10+ cells increased in still symptomatic GWVs with a higher level of statistical significance (Table 2).

Since allergic disease is associated with a Th2 type immune response, it was important to exclude pre-existing allergy as a cause of our observations. Self-reported hay fever was more frequent in the sGWV group than in the wGWVs, consistent with our previous demonstration of high levels of self-reporting of all forms of illness in this group. Given the unreliability of self-reporting in this cohort, therefore, we measured serum IgE levels as a surrogate marker of Th2-mediated hypersensitivity. Levels were not statistically different in sGWVs and wGWVs, and there was no correlation between IgE levels and IL-4+, IL-10+ or IFN-γ+ CD4 T cells in any study groups. We therefore conclude that IgE-mediated hypersensitivity is not the underlying cause of the high levels of IL-4+ and IL-10+ cells we observed.

2.3 Vaccine effects on Th1/Th2 balance in Gulf War veterans

Our previous research indicated a weak association between multiple vaccines and symptoms, but not disability measured on the SF-36 physical functioning scale. Therefore it is not surprising that vaccine status in sGWVs and wGWVs did not differ in the present study. We assessed the association between multiple vaccines and physical symptoms in the sample on whom we had immune data, and were unable to find an association between ill-health and multiple vaccines in this sample.

Gulf war veterans received variable numbers of vaccines. No single agent, including anthrax and plague vaccines given for protection against possible biological warfare, was associated with significant Th1/Th2 shift. However, there was a significant association between reducing levels of IFN-γ+ Th1 cells and increasing total vaccine exposure ($p<0.05$). Trends for increasing levels of IL-4+ and IL-10+ cells were also observed but failed to reach statistical significance.

Key Research Accomplishments

- This is the first systematic study of which we are aware to examine intracellular Th1 and Th2 cytokine expression in a large cohort of patients from the following groups: Sick GWVs, well GWVs and sick Bosnia/Era veterans.

- Sick GWVs are characterised by the presence of significantly expanded populations of CD4 cells which, without stimulation, secrete IL-4, IFN-γ, IL-2 and IL-10. This is the so-called Th0 pattern, and these cells are likely to be recently activated.

- In contrast, after stimulation, sick GWVs demonstrate high levels of memory cells which secrete IL-4. Additional analyses have confirmed that these cells do not express IFN-γ and are therefore true Th2 cells.

- Secretion of IL-10 (considered a Th2 or Treg cytokine) by memory cells is elevated most markedly in sick veterans, irrespective of deployment.

- The Th2 pattern of cytokine secretion in symptomatic GWVs is not related to pertussis, or to any single vaccine agent, including anthrax.

- A weak but significant association between reducing levels of IFN-γ secreting memory cells and multiple vaccination was observed. This would support the Rook and Zumla hypothesis that multiple vaccines may induce Th2 shift. However, since vaccine exposure in the GWVs (sick and well) was similar, we are unable to confirm a causative link between vaccines, Th2 shift and multi-symptom illness.

- The presence of high levels of IL-10 producing memory cells in sick veterans is an important finding, and may have bearing on the efficacy of such procedures as multiple vaccination, since IL-10 is noted for its ability to suppress immune responses.

In summary, these results demonstrate a complex pattern of abnormalities. Sickness amongst GWVs is associated with ongoing Th0 immune activation and evidence of previous Th2 polarisation amongst memory CD4 cells. Th2 polarization is linked to multiple vaccine exposure, albeit weakly. Sickness typical of GW deployment is associated with induction of memory CD4 cells secreting the regulatory cytokine IL-10.

Reportable Outcomes

These data are currently under review by a journal.

Conclusions

Our results demonstrate unusual but consistent changes in cytokine secretion in GWVs. Certainly it can be concluded that their cytokine profiles are not normal. Of particular interest is the Th2 shift in sick GWVs, and its association with multiple vaccines, and also the evidence for induction of regulatory T cells during multi-symptom illness.

Suggestions for future research

The following studies may be informative as to the mechanisms and relevance of our findings:

1. A prospective study of recruits undergoing routine multiple *vs* infrequent vaccination to examine (a) whether multiple vaccination is accompanied by a Th2 shift (in the absence of the stress of deployment) and (b) whether immunity to the vaccines is compromised by multiple vaccination
2. A study of the cellular and molecular mechanisms of immune deviation we observed. This would focus on the dendritic cell as the arbiter of Th1/Th2 decisions by T cells during priming, and the effect of multiple and single vaccine agents, as well as corticosteroids to represent "stress".
3. A study of the recall T cell response to vaccine agents in selected veterans characterised in our study above. This would establish whether vaccine efficacy is compromised by the presence of high levels of regulatory T cells.

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Table 1 Expression of intracellular cytokine production by CD4+ cells within lymphocyte population in all cohorts

	% IFN - γ	% IL - 4	% IL - 2	% IL - 10
	Resting	Resting	Resting	Resting
BI+EI	1.28 (1.00)	2.06 (0.88)	0.97 (0.83)	1.56 (1.12)
GW	1.30 (1.02)	2.10 (0.99)	1.02 (0.89)	1.57 (1.06)
GI	1.72 (1.19)	3.03 (1.51)	1.46 (1.00)	1.71 (1.03)

Mean (SD) percentage of total lymphocytes positive for different cytokines and CD4 under resting (ie unstimulated) and mitogen-activated conditions. BI, ill Bosnia veterans; EI, ill era controls; GW, well Gulf veterans; GI, ill Gulf veterans.

Appendix I

Table 2 Expression of intracellular cytokine production by CD8+ cells within lymphocyte population in all cohorts

	% IFN - γ		% IL - 4		% IL - 2		% IL - 10	
	Resting	Activated	Resting	Activated	Resting	Activated	Resting	Activated
BI+EI	0.91 (0.80)	9.37 (5.08)	1.26 (0.79)	1.01 (0.78)	0.60 (0.58)	2.93 (1.69)	1.04 (0.73)	1.48 (0.96)
GW	1.21 (0.91)	8.72 (3.92)	1.55 (0.99)	1.18 (0.75)	0.95 (0.80)	2.83 (1.73)	1.44 (1.13)	1.24 (0.78)
GI	1.19 (0.94)	8.73 (3.76)	1.69 (0.93)	1.48 (0.96)	0.79 (0.65)	2.83 (1.58)	1.13 (0.96)	1.41 (1.06)

Mean (SD) percentage of total lymphocytes positive for different cytokines and CD8 under resting (ie unstimulated) and mitogen-activated conditions. BI, ill Bosnia veterans; EI, ill era controls; GW, well Gulf veterans; GI, ill Gulf veterans.

Table 3 P values calculated by Mann – Whitney test between cohorts for CD4+ cells

Comparison of cohorts	IL-4	IFN-γ	IL-2	IL-10
	Resting	Resting	Resting	Resting
BI+EI vs GW	0.8784	0.8906	0.6645	0.88
BI+EI vs GI	**0.0002**	**0.0245**	**0.0017**	0.47
GW vs GI	**0.0007**	**0.0359**	**0.0054**	0.49

Statistically significant differences are given in bold. BI, ill Bosnia veterans; EI, ill era controls; GW, well Gulf veterans; GI, ill Gulf veterans.

Table 4 P values calculated by Mann – Whitney test between cohorts and lab control in CD8+ cells

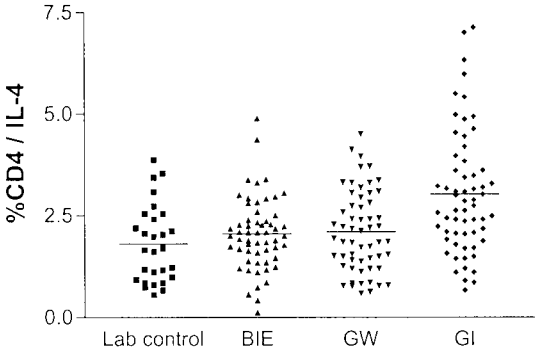
Comparison of cohorts	IL-4		IFN-γ		IL-2		IL-10	
	Resting	Activated	Resting	Activated	Resting	Activated	Resting	Activated
BI+EI vs GW	0.1427	0.1855	0.0930	0.7307	0.0179*	0.6044		
BI+EI vs GI	0.0115*	0.0041*	0.0522	0.8548	0.0533	0.9304		
GW vs GI	0.3250	0.1552	0.8250	0.8995	0.4302	0.5893		

Statistically significant differences are given in bold. BI, ill Bosnia veterans; EI, ill era controls; GW, well Gulf veterans; GI, ill Gulf veterans. For technical reasons, IL-10 data were not available on CD8+ T cells.

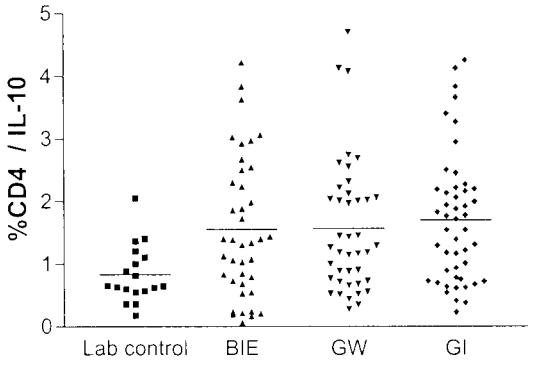
UNSTIMULATED

Appendix V

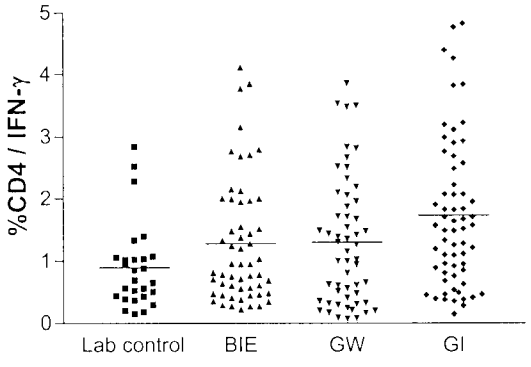
CD4+ / IL-4 unstimulated



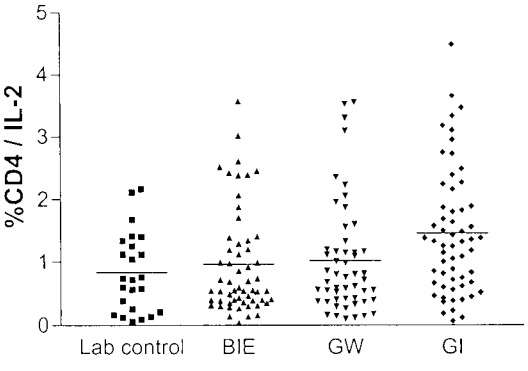
CD4+ / IL-10 unstimulated



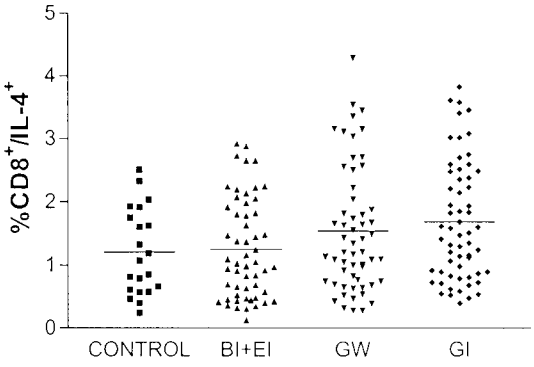
CD4+ / IFN-γ unstimulated



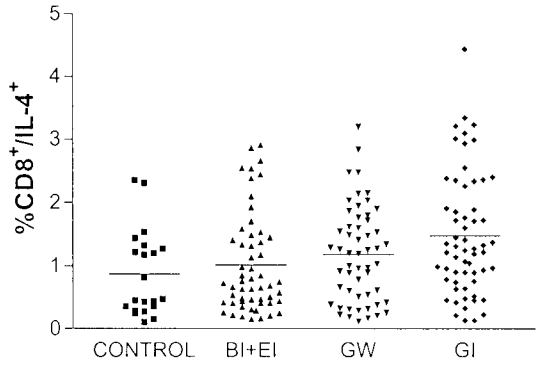
CD4+ / IL-2 unstimulated



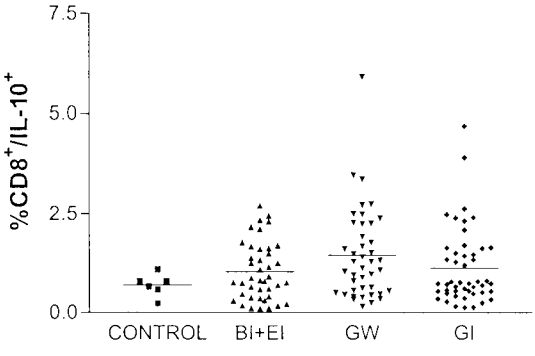
CD8/lymph/IL-4 - unstimulated



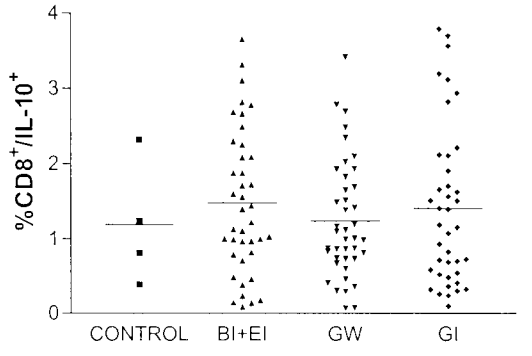
CD8/lymph/IL-4 - stimulated



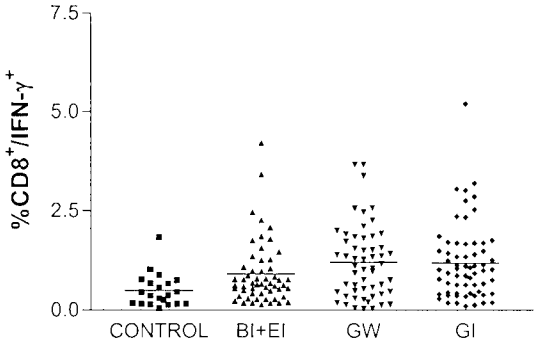
CD8/lymph/IL-10 - unstimulated



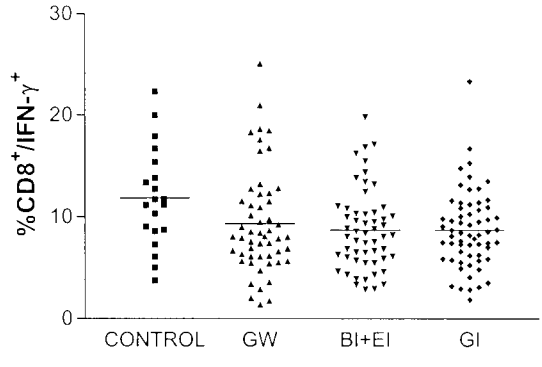
CD8/lymph/IL-10- stimulated



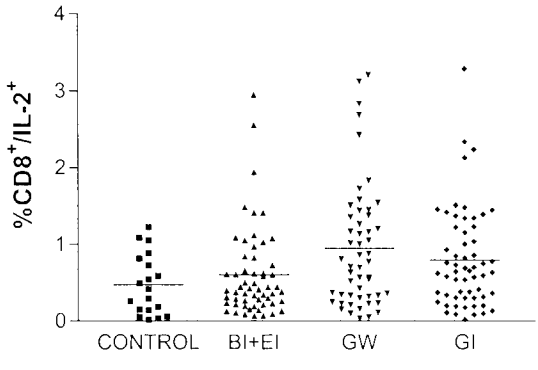
CD8/lymph/IFN-γ - unstimulated



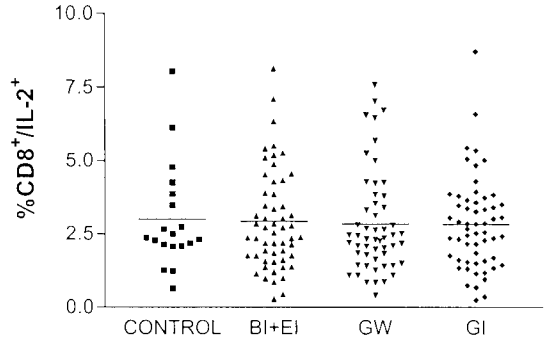
CD8/lymph/IFN-γ - stimulated



CD8/lymph/IL-2 - unstimulated



CD8/lymph/IL-2 - stimulated



Appendix VII

Legends to figures

Figure 1
Percentage cytokine positive CD4+ T cells identified by intracellular cytokine staining (IL-4, IL-10, IFN-γ and IL-2) of whole peripheral blood (cultures left unstimulated). Horizontal bars represent means. For statistical comparisons, see Tables 1-4, Appendices I-IV. Values are given for sick veterans of the Bosnia campaign/non-deployed service staff (Era controls; BI+EI), well veterans of the Gulf War (GW) and ill veterans of the Gulf War (GI). A small number of healthy laboratory controls are included for comparative purposes.

Figure 2
Percentage cytokine positive CD8+ T cells identified by intracellular cytokine staining (IL-4, IL-10, IFN-γ and IL-2) of whole peripheral blood. Panels on the left are from cultures left unstimulated and on the right from cultures activated with PMA and ionomycin. Horizontal bars represent means. For statistical comparisons, see Tables 1-4, Appendices I-IV. Values are given for healthy laboratory controls, sick veterans of the Bosnia campaign/non-deployed service staff (Era controls; BI+EI), well veterans of the Gulf War (GW) and ill veterans of the Gulf War (GI). A small number of healthy laboratory controls are included for comparative purposes.

T helper 2 type immune activation in Gulf War veterans with multi-symptom illness

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Summary

Background The aetiology of Gulf War–related illness is unclear, but one theory is that several factors, including multiple vaccinations given under the stress of deployment, promote a T helper 2 (Th2) shift in immune responsiveness, resulting in the multi-symptom illness observed [1]. In support of this hypothesis, epidemiological evidence indicates that multiple vaccinations are a significant risk factor for Gulf War–related illnesses. We therefore measured intracellular production of interferon-γ and interleukin-2 (IFN-γ and IL-2; Th1 cytokines) and IL-4 and IL-10 (Th2 cytokines) by CD4 lymphocytes from Gulf War veterans (with and without multi-symptom illness), and non-Gulf service personnel, to investigate the relationship between Th1/Th2 balance, multi-symptom illness, and the administration of multiple vaccines.

Methods Employing a nested case control study design within a large epidemiological survey, we were able to compare symptomatic Gulf War veterans (sGWV) with well Gulf War veterans (wGWV), and a control group of multi-symptomatic veterans who served in Bosnia or were non-deployed military personnel of the same era (sBEV). Th1/Th2 balance was measured on fresh whole blood samples activated with mitogens, by flow cytometric quantification of CD4 T helper cell secretion of IFN-γ, IL-2, IL-4 and IL-10.

Findings We found evidence of Th2 skewing in sGWV. Levels of IL-4 secreting CD4 T cells in sGWV were higher compared with wGWV ($p<0.05$) and sBEV ($p<0.05$) in whom levels were similar. Levels of IL-10 producing CD4 cells were similar in sGWV and sBEV but were higher than in wGWV ($p<0.005$ and $p<0.05$, respectively). Levels of Th1 cytokine producing cells were similar in all groups. However, amongst Gulf War veterans, we found a significant trend for reduced levels of the Th1 cytokine IFN-γ and non-significant trends for increased levels of the Th2 cytokines IL-4 and IL-10 with increasing numbers of vaccines administered.

Interpretation These data show that multi-symptom illness in Gulf War veterans is characterised by biased generation of cells secreting the prototypic Th2 cytokine, IL-4. Secretion of IL-10, a less specific Th2 marker, was associated with multi-symptom illness irrespective of deployment. Multiple vaccine exposure was associated with evidence of Th1/Th2 imbalance in favour of Th2, but since vaccine exposure in the symptomatic and well Gulf war veterans we studied was similar, we are unable to confirm a causative link between vaccines, Th1/Th2 balance, and illness.

Key words: Gulf War, cytokines, Th1/Th2 balance, Gulf War-related illness.

Introduction

Almost 53,000 UK service personnel took part in the 1990-91 Persian Gulf war. Following their return, many Gulf War veterans sought medical advice for symptoms they felt were related to their wartime service. Our previous large epidemiological study showed that Gulf War veterans had markedly increased rates of ill health when compared with non-deployed service personnel and veterans of peacekeeping duties in Bosnia, although this and most studies have failed to find a discrete, specific syndrome [2-5]. The most frequent complaints amongst veterans are of fatigue, rashes, joint and/or muscle pain, neuropsychiatric complaints, shortness of breath, sleep disturbances, and gastrointestinal problems [3]. Various aetiologies have been proposed, including post-traumatic stress disorder [6], possible lead absorption from oil-well fire smoke, the diesel stoves used to heat tents, depleted uranium [7], anti-nerve gas drugs [8], chemical weapons [9], insect repellents [10] and multiple vaccinations [11].

In this journal, Rook and Zumla proposed that Gulf War illnesses were the result of a T helper 2-biased immune response [1]. The T helper1/helper2 (Th1/Th2) paradigm of immune responsiveness has been used to further understanding of a number of disease states associated with immune dysfunction, such as allergy [12] and autoimmune disease [13]. Th2-associated disorders such as hypersensitivity [12] and mood changes [14] are similar to symptoms reported by some veterans. Rook and Zumla argued that Gulf War veterans were exposed to several stimuli that strongly favour a T helper 2-biased immune response. First, veterans received multiple vaccinations administered within a short space of time, equivalent to an excessive antigen load that is known to deviate the immune response towards Th2 [15,16]. Second, biological warfare vaccines were given in stressful, near-combat conditions after deployment. Stress-associated glucocorticoid hormones deviate the immune response towards Th2 under experimental conditions [17-20]. Third,

although natural infection with *Bordetella pertussis* and its whole cell-derived vaccine promotes a strong Th1 response, paradoxically the acellular vaccine component of pertussis toxin used as an adjuvant by United Kingdom forces in the Gulf can cause Th2 deviation [21,22].

Recently, the Rook and Zumla hypothesis received some support from an extension of our epidemiological study, showing that receiving vaccinations after deployment to the Gulf, and receiving multiple vaccinations, were associated with multi-symptom illness in the Gulf War veteran cohort [11]. To explore the link between cytokine imbalance and illness, we studied cytokine profiles in Gulf War veterans with and without multi-symptom illness, and with a range of vaccine exposures. To examine whether cytokine imbalance was peculiar to Gulf service, we also studied a cohort of non-deployed service personnel and veterans of peacekeeping duties in Bosnia who displayed similar multi-symptom ill health.

Methods

Patients and study design

Blood samples were obtained from volunteers attending the Gulf War Illness Research Unit at GKT Medical School who were participants in our cross-sectional stage II analysis, following on from the questionnaire based stage I epidemiological study previously reported [3,11,23]. The current study included 57 symptom free veterans (well Gulf War veterans - wGWV) and 63 veterans who were symptomatic (sGWV). To examine effects specifically associated with Gulf War related illness the study also included 58 military personnel, either veterans of peace keeping duties in Bosnia (n=20) or in military service in 1991 but not actually deployed to the Gulf War (n=38), who also displayed multi-symptom illness (symptomatic Bosnia/era control group, sBEV). Illness status was assigned according to the stage I analysis. In the absence of an accepted or meaningful classification of Gulf War-related multi-symptom illness, we chose a case definition based on symptomatic and functional ill health. Symptomatic individuals (sGWV and sBEV) were defined as those who had scored 72.2 or less on the SF-36 physical functioning subscale. This was the cut-off value for the lowest 10^{th} centile of the distribution of the SF-36 PF in the stage I analysis of era personnel. The study was performed blindly using coded blood samples from the four defined groups and was approved by the Institute's Ethical Review Committee (LREC 96-172a).

Exposure to vaccines was recorded in two ways. Firstly, with the individual's consent, we attempted to trace personal medical records for study participants to gain details of recorded exposure to vaccines in the period June 1990 to February 1991. From this we were able to calculate the total vaccines received. Secondly, respondents to the original survey completed a questionnaire which included details about (1) whether the serviceman had his vaccine record, (2) how many and which vaccines he received in the two months prior to deployment, and (3) how many and which vaccines he received during deployment. From the replies to these questions we

calculated the total number of vaccines received. We assumed that the vaccine records would be more accurate than reported vaccines, so in individuals with vaccine records (95/120 GWV) records were used, and missing values were substituted by reported vaccine exposure.

As depression was more common in the sGWV group, and has been associated with a range of immune changes, we measured depression on the Beck Depression Inventory (BDI) when participants attended for their detailed medical assessment.

Cell culture and intracellular cytokine staining

Flow cytometry was used to measure intracellular cytokine production by CD4 T lymphocytes. Heparinised venous whole blood taken from all subjects at approximately the same time of day to avoid the effect of diurnal variation, was mixed with tissue culture medium (TCM; RPMI 1640, 10% foetal calf serum (FCS), 2mM L-Glutamine, 100IU/ml penicillin, 100μg/ml streptomycin, Life Technologies, Paisley, Scotland) in the proportion 1:3.5 TCM and 1ml then supplemented with mitogen to 5ng/ml final concentration of M phorbol 12-myristate 13-acetate (PMA) and 745ng/ml of ionomycin (Sigma Chemical Co, Poole, UK). Protein secretion inhibitors brefeldin A (5μg/ml) and monensin (2.08mg/ml; both Sigma) were added and blood cultured at 37°C for 16 hours in 5% CO_2.

Cells were harvested, washed and stained with PerCP conjugated anti-human CD4 monoclonal antibodies (mAb) (Becton Dickinson, San Jose, CA), then washed twice with phosphate-buffered saline (PBS) containing 5% FCS and 0.01% sodium azide and intracellular cytokine staining carried out as suggested by the manufacturer (Fix&Perm Permeablilization Kit, Caltag Lab, Burlingame, USA). In brief, the cells were fixed with Reagent A and incubated for 15 min at room temperature in the dark. After washing twice permeabilizing Reagent B was added along with appropriate PE-conjugated anti-cytokine (IL-2, IFN-γ, IL-4, IL-10) mAbs or isotype-

matched control mAbs (all Becton Dickinson) and incubated in the dark at 4°C for 30 minutes. Finally, the stained cells were washed and suspended in 200μl of PBS containing 0.01% sodium azide for flow cytometry analysis. Additional studies were carried out to examine co-expression of IL-4 and IFN-γ using CD3-FITC, CD4-PerCP, IFN-γ-PE and IL-4–APC mAbs.

Lymphocytes were gated on the basis of forward and side scatter properties and fluorescent channel dot plot quadrant statistics set on the basis of corresponding isotype-matched control mAbs to determine the frequencies of CD4 T cells producing IL-2, IFN-γ, IL-4 and IL-10. Contamination by CD14+ monocytes in the gated CD4 population was routinely assessed as <1%. Stained cells were analyzed on a FACSCalibur cytometer using CELLQuest software (Becton Dickinson, San Jose, CA) and cytokine positive cells expressed as percent positive of total lymphocytes.

Serum IgE levels

Serum IgE was measured by latex enhanced laser nephelometry using specific antisera and a Behring Laser Nephelometer II as recommended by the manufacturers.

Statistical analysis

Statistical analyses were performed using STATA version 6.0. We determined that all the cytokine data were normally distributed, and therefore used t-tests to assess differences between the sGWV and wGWV. Because our previous research had shown that multiple vaccines were associated with ill-health in GWVs, we reasoned that any immune changes could be explained by differences in vaccine exposure between the two groups. We therefore entered vaccine exposure into a multiple regression model, with cytokine level as the dependent variable, to determine whether any association between cytokine levels and illness status could be explained by vaccine exposure. We also entered depression score (from the BDI) into the model to determine whether

depressive symptoms were a potential confounder. Results are expressed as mean differences between groups with 95% confidence intervals.

Results

Th1/Th2 balance in Gulf War veterans

We found evidence for a shift towards Th2 immunity in symptomatic GWVs. Mean levels of IL-4 producing Th2 cells were higher in symptomatic GWVs than in well GWVs ($p<0.05$) and symptomatic BEVs ($p<0.05$) (figure 1, table 1). Mean levels of IL-10 producing CD4 T cells were similar in symptomatic GWVs and sBEVs, but were higher in both than well GWVs ($p=0.001$ and $p<0.05$, respectively). In contrast, levels of IFN-γ and IL-2 producing Th1 cells were similar in all study groups.

To confirm that IL-4 producing CD4 T cells were indeed Th2 and not Th0 (Th0 cells secrete IL-4 and IFN-γ), a subset of cases (n=40) was also analysed by 4-colour flow cytometry for co-secretion of IL-4 and IFN-γ. In all cases, over 85% of IL-4+ CD4 T cells did not secrete IFN-γ, confirming the predominance of Th2 cells. There was no correlation between levels of IL-4 producing and IL-10 producing CD4 T cells in sGWVs, suggesting the existence of two distinct, expanded populations of effector memory cells.

Illness effects on Th1/Th2 balance in Gulf War veterans

Our data suggest that sickness amongst GWVs is associated with elevated levels of IL-4+ Th2 cells. To examine the effects of illness on Th1/Th2 balance, we compared results between symptomatic and well GWVs. No single symptom was significantly associated with Th2 skewing but when levels of IL-4+ and IL-10+ cells were controlled for mood (assessed using the BDI), significance levels were reduced, becoming non-significant in the case of IL-4 (Table 1). These results suggest that expansion of IL-4+ and IL-10+ Th2-type cells is associated with depression, but this is not a sufficient explanation of the observed findings.

During the period between the initial stage I questionnaire based study, and clinical assessment carried out for stage II, which included the blood sampling for the current study, 25 (42%) sGWVs showed sufficient improvement in their SF36 physical functioning score that they no longer fulfilled our criteria to be considered "ill", and 3 (6%) of the wGWVs had experienced a deterioration in their SF36 physical functioning and were considered cases. When these cross-overs were taken into account, the association between illness and Th1/Th2 balance also changed. Although mean levels of IL-4+ cells remained higher in symptomatic GWVs, this difference was no longer significant (Table 2). However, mean levels of IL-10+ cells increased in still symptomatic GWVs with a higher level of statistical significance (Table 2).

Since allergic disease is associated with a Th2 type immune response, it was important to exclude pre-existing allergy as a cause of our observations. Self-reported hay fever was more frequent in the sGWV group than in the wGWVs, consistent with our previous demonstration of high levels of self-reporting of all forms of illness in this group [3]. Given the unreliability of self-reporting in this cohort, therefore, we measured serum IgE levels as a surrogate marker of Th2-mediated hypersensitivity. Levels were not statistically different in sGWVs and wGWVs, and there was no correlation between IgE levels and IL-4+, IL-10+ or IFN-γ+ CD4 T cells in any study groups. We therefore conclude that IgE-mediated hypersensitivity is not the underlying cause of the high levels of IL-4+ and IL-10+ cells we observed.

Vaccine effects on Th1/Th2 balance in Gulf War veterans

Our previous research indicated a weak association between multiple vaccines and symptoms, but not disability measured on the SF-36 physical functioning scale. Therefore it is not surprising that vaccine status in sGWVs and wGWVs did not differ in the present study. We assessed the association between multiple vaccines and physical symptoms in the sample on whom we had

immune data, and were unable to find an association between ill-health and multiple vaccines in this sample.

Gulf war veterans received variable numbers of vaccines. No single agent, including anthrax and plague vaccines given for protection against possible biological warfare, was associated with significant Th1/Th2 shift. However, there was a significant association between reducing levels of IFN-γ+ Th1 cells and increasing total vaccine exposure ($p<0.05$). Trends for increasing levels of IL-4+ and IL-10+ cells were also observed but failed to reach statistical significance.

Discussion

The present study was designed to examine the hypothesis put forward by Rook and Zumla that Gulf war-related multi-symptom illness resulted from a shift in immune balance towards Th2, as a result of multiple vaccines, use of pertussis as adjuvant, and biological warfare vaccines given under conditions of operational stress [1].

The first proposal of the "Th2 hypothesis" is that Gulf War related illness is associated with a Th2 shift in cytokine balance. We found that Th2 cells secreting the prototypic cytokine IL-4 were elevated in symptomatic Gulf War veterans, compared with the other study groups. The second proposal is that the Th2 shift results from exposure to multiple vaccines, pertussis, or stress. We found a weak but significant relationship between multiple vaccines and cytokine imbalance in favour of Th2 in Gulf War veterans. A shift in immune balance in favour of Th2 was suggested (a) by the significant decline in IFN-γ+ Th1 cells with increasing vaccine numbers (Th1 and Th2 cells are mutually antagonistic) and (b) the trend towards increasing numbers of IL-4 secreting Th2 cells with increasing vaccine numbers. Exposure to stress is difficult to quantify, but we were able to exclude pertussis or any other single vaccine agent, including anthrax, as being associated with Th2 shift.

The final key element of the "Th2 hypothesis" was that a Th2 shift in immunity gave rise to multi-symptom illness of the type seen in Gulf War veterans. We could not find any strong association between single symptoms and Th2 shift. Our data suggest that Th2 shift may be associated with symptoms of depression, but we are unable to decipher cause and effect. Studies by Maes and co-workers suggest a strong relationship between depression arising as a direct result of cytokine therapy for cancer and chronic virus infection and some markers of immune activation, especially serum IL-10 levels [14]. Prospective studies of service personnel will be

required to establish whether immune imbalances such as those we have observed cause depression or are secondary to it.

Since our previous epidemiological study linked illness with multiple vaccines, and we have shown an association between the latter and Th2 shift, are we now able to "close the loop" between vaccine exposures, Th2 shift and illness? The answer is that we cannot, mainly because the vaccine effects on illness we reported are weak. The considerable time between the original exposure to stimuli capable of disturbing the Th1 and Th2 immune balance and our analysis adds to the complexity of the relationships we have uncovered. In addition, our random selection of cases for stage II analysis has resulted in similar levels of vaccine exposure in both well and symptomatic Gulf War veterans.

The association between multi-symptom illness, seen in Gulf War veterans and non-deployed/Bosnia veterans, and elevated levels of IL-10 producing cells is particularly striking. The health of some previously symptomatic Gulf War veterans improved between stages I and II of our studies, and this improvement was accompanied by a strengthening of the association between illness and levels of IL-10 producing CD4 cells in those who remained sick. IL-10 was originally described as a cytokine produced by Th2 cells, although it is now apparent that other cells can produce IL-10, including regulatory T cells [24]. We were unable to demonstrate a clear correlation between IL-4 and IL-10 producing cells, suggesting that these constitute two distinct populations of effector cells in symptomatic veterans. IL-10 mediates potent anti-inflammatory effects, earning it the term "suppressor of systemic pathology". In animal models IL-10 protects against severe inflammatory pathology associated with infection, and contributes to impaired clearance of infectious agents such as *Leishmania* [24].

Irrespective of the mechanism of induction of high levels of IL-10 producing CD4 cells, our finding raises important considerations in relation to vaccination. For example, high IL-10 levels during the deployment period, when vaccination is being carried out, could have unwanted effects on the induction of protective immunity.

It will be important in future studies to delineate the mechanisms that lead to Th2 shift in symptomatic Gulf War soldiers. We propose a model centred around the dendritic cell (DC), the antigen presenting cell largely responsible for instructing the Th1/Th2 balance of T cells as they undergo activation from the naïve state [25]. In their immature form, DCs capture antigen in the periphery, transport it to the regional lymph node to activate T cells appropriately, and themselves undergo a maturation process, acquiring surface molecules and cytokine secretion patterns, the balance of which shape the Th1/Th2 immune repertoire. Numerous factors are known to influence this maturation and instruction process. Antigen overload (c.f. multiple vaccinations) skews the immune response towards Th2 [15,16], possibly as a result of interference in DC maturation. In addition, as a response to stress the hypothalamic-pituitary-adrenal system releases glucocorticoids that promote IL-4 and IL-10 producing T cells [20]. IL-10 blocks DC maturation, and immature DCs promote Th2 responses [26]. The sum of these effects could be a Th2 dominated immune system. However, prospective studies will be required to establish whether Th2 skewing causes multi-symptom illness, is an unrelated finding, or arises secondarily to influences of the neuro-immuno-endocrine axis.

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Figure Legends

Figure 1

T helper 2 shift in cytokine production in Gulf War veterans with multi-symptom illness. Graph shows percentage of CD4 T cells staining for Th2 (panels A and B, IL-4+ and IL-10+ cells respectively) and Th1 (panels C and D, IFN-γ+ and IL-2+ cells, respectively) in Bosnia/Era veterans with multi-symptom illness (sBEV) and Gulf War veterans with and without multi-symptom illness (sGWV and wGWV, respectively).

Table 1: Relationship between Gulf illness status as defined from Stage I questionnaire based study and levels of cytokine producing cells.

Th1/Th2 cells	Symptomatic GWVs (%)	Well GWVs (%)	mean difference (95% CI)	mean difference (95% CI) corrected for BDI	mean difference (95% CI) corrected for vaccines
Th2					
IL-4	4.44	3.64	0.80 (0.04, 1.56)	0.68 (-0.21, 1.58)	0.70 (-0.05, 1.46)
			p=0.04	p=0.13	p=0.07
IL-10	4.47	3.22	1.25 (0.50, 2.00)	1.06 (0.22, 1.90)	1.21 (0.42, 2.00)
			p=0.001	**p=0.015**	**p=0.003**
Th1					
IFN-γ	11.6	11.9	-0.26 (-2.04, 1.52)	-0.99 (-3.01, 1.10)	-0.55 (-2.37, 1.26)
			p=0.8	p=0.35	p=0.5
IL-2	17.3	16.7	-0.58 (-2.91, 1.75)	-1.06 (-3.60, 1.49)	-0.87 (-3.29, 1.56)
			p=0.6	p=0.4	p=0.5

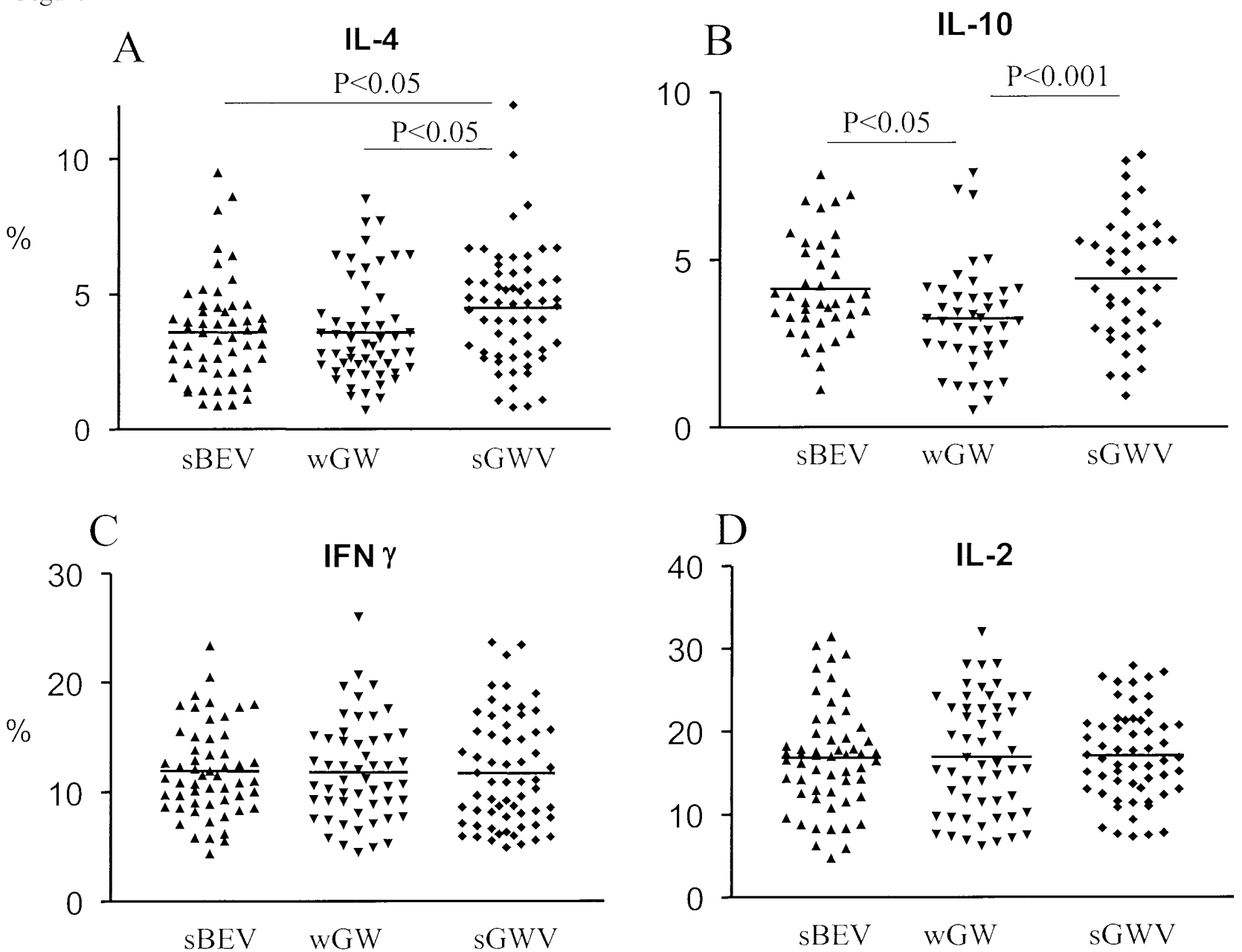
GWV, Gulf War veteran; BDI, Beck depression index

Table 2: Relationship between Gulf illness status as defined from Stage II (ie taking into account cross overs) and levels of cytokine producing cells.

Th1/Th2 cells	Symptomatic GWVs (%)	Well GWVs (%)	mean difference (95% CI)	mean difference (95% CI) corrected for BDI	mean difference (95% CI) corrected for vaccines
Th2					
IL-4	4.33	3.93	0.40 (-0.43, 1.23) p=0.3	0.14 (-0.79, 1.07) p=0.8	0.89 (-0.06, 1.85) p=0.06
IL-10	5.08	3.20	1.88 (1.12, 2.63) **p<0.001**	1.79 (0.96, 2.62) **p<0.001**	1.86 (0.95, 2.76) **p<0.001**
Th1					
IFN-γ	12.0	11.6	-0.46 (-1.43, 2.34) p=0.6	-0.10 (-2.261, 2.05) p=0.9	-0.43 (-2.61, 1.74) p=0.7
IL-2	17.4	16.4	-0.99 (-3.50, 1.52) p=0.4	-1.58 (-4.21, 1.05) p=0.4	-1.79 (-4.64, 1.05) p=0.2

GWV, Gulf War veteran; BDI, Beck depression inventory

Figure 1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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 8 Jan 2003

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed. Request the limited distribution statement for the enclosed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
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